

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/00, 7/00, 5/00 C07H 15/12	A1	(11) International Publication Number: WO 90/12094 (43) International Publication Date: 18 October 1990 (18.10.90)
(21) International Application Number: PCT/US90/01716 (22) International Filing Date: 4 April 1990 (04.04.90) (30) Priority data: 311,217 5 April 1989 (05.04.89) US (71) Applicant: THE UNITED STATES OF AMERICA as represented by THE SECRETARY, U.S. DEPARTMENT OF COMMERCE [US/US]; 5285 Port Royal Road, Springfield, VA 22161 (US). (72) Inventors: ICHO, Tatco ; 2-28-Sonechi Chofu, Tokyo 182 (JP). WICKNER, Reed, B. ; 9010 Bradgrove Drive, Bethesda, MD 20817 (US).		(74) Agents: HOLMAN, John, Clarke et al.; Fleit, Jacobson, Cohn, Price, Holman & Stern, The Jenifer Building, 400 Seventh Street, N.W., Washington, DC 20004 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: A CLONE OF DOUBLE-STRANDED RNA VIRUS AND APPLICATIONS THEREOF (57) Abstract A cDNA clone of the double-stranded RNA genome of a yeast virus and various applications thereof are described.		

+ See back of page

NUC 24467

DESIGNATIONS OF "DE"

Until further notice, any designation of "DE" in any international application whose international filing date is prior to October 3, 1990, shall have effect in the territory of the Federal Republic of Germany with the exception of the territory of the former German Democratic Republic.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MR	Mauritania
BE	Belgium	GA	Gabon	MW	Malawi
BF	Burkina Faso	GB	United Kingdom	NL	Netherlands
BG	Bulgaria	HU	Hungary	NO	Norway
BJ	Benin	IT	Italy	RO	Romania
BR	Brazil	JP	Japan	SD	Sudan
CA	Canada	KP	Democratic People's Republic of Korea	SE	Sweden
CF	Central African Republic	KR	Republic of Korea	SN	Senegal
CG	Congo	LI	Liechtenstein	SU	Soviet Union
CH	Switzerland	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany, Federal Republic of	MC	Monaco	US	United States of America
DK	Denmark				

A CLONE OF DOUBLE-STRANDED RNA VIRUS
AND APPLICATIONS THEREOF

This is a continuation in part of the pending application Serial Number 07/169,486 filed March 17, 1988.

The present invention is related generally to the field of genetic engineering. More particularly, the present invention is related to providing a cDNA clone of the double-stranded RNA genome of a yeast virus and various applications of the clone.

Viruses use a wide variety of strategies to replicate and differentially express the proteins they encode. Retroviruses, double-stranded RNA (dsRNA) viruses and (+) strand RNA viruses including Picornaviruses and Togaviruses, have in common their use of genomic (+) strand RNA as a template for replication and as a message for protein synthesis. Thus, while DNA viruses and (-) strand RNA viruses use splicing of mRNA to produce, in different amounts, proteins sharing part of their sequence (Livingston et al, 1985, Replication of Papovaviruses. In Virology, B.N. Fields, ed., New York: Raven Press, pp. 393-410; Horwitz, 1985, Adenoviruses and their replication. In Virology, B. N. Fields, ed, New York: Raven Press, pp. 433-476; Kingsbury, 1985, Orthomyxo- and Paramyxoviruses and their replication. In Virology, B.N. Fields, ed., New York: Raven Press, pp. 1157-1178), splicing is unknown among (+) strand RNA viruses and dsRNA viruses, probably because the spliced RNA would be packaged and replicated leading to the accumulation of defective viruses. Retroviruses splice their genomic RNA to

make the env protein, but the spliced RNA lacks the Psi sequence necessary for packaging (Watanabe et al, 1983, Mol. Cell. Biol. 3:2241-2249; Mann et al, 1983, Cell 33:153-159; Markowitz et al, 1988, J. Virol 62:1120-1124). In addition, retroviruses use ribosomal frameshifting to make a large amount of the gag protein and a small amount of the gag-pol fusion protein (Jacks et al, 1988, Cell 55:447-458). Murine leukemia virus (Varms, 1988, Science 240:1427-1435) and two α viruses (Strauss et al, 1983, Proc. Natl. Acad. Sci. USA 80:5271-5275) use nonsense suppression in the same way. The rates of transcription and translation of different reovirus dsRNA segments vary over a range of more than 20-fold to give overall rates of expression that vary over 400-fold (Joklik, 1981, Microbiol Rev. 45:483-501).

L-A is a dsRNA virus of Saccharomyces cerevisiae. L-A replicates by a conservative mechanism, with (+) strands made by transcription of dsRNA and (-) strands then made by copying the (+) strands to form dsRNA again (sequential synthesis) (Newman et al, 1981, J. Virol 38:263-271; Sclafani et al, 1984, Mol. Cell. Biol. 4:1618-1626; Newman et al, 1986, The replication of dsRNA. In Extrachromosomal Genetic Elements in Lower Eukaryotes, R. B. Wickner, A. Hinebusch, A. M. Lambowitz, I. C. Gunsalus, A. Hollaender, eds., New York: Plenum Press, pp. 173-187; Fujimura et al, 1986, Proc. Natl. Acad. Sci. USA 83:4433-4437). (+) single-stranded RNA (ssRNA) is packaged in a new coat to form new viral particles

(Fujimura et al, 1986, supra; Fujimura et al, 1987, Mol. Cell. Biol. 7:420-426). Both (+) and (-) strands are synthesized within viral particles, and because the size of the heads appears to be designed to contain one L-A molecule (4.6 kb), dsRNAs less than half the size of L-A replicate within the head until they fill it with multiple copies.

The 4.6 kb L-A dsRNA genome with a coding capacity of about 180 kDa encodes both the 80 kDa major coat protein (over 100 molecules per viral particle) and a 180 kDa minor viral protein (about 1 molecule per particle). The 180 kDa protein shares immunodeterminants with the major coat protein and, unlike the major coat protein, has ssRNA-binding activity which could play a role in both packaging and replication. L-A carries several genetic activities, called [HOK], [NEX], [EXL] and [B] defined by the interactions of L-A with M dsRNA (see review by Wickner, 1986, Ann. Rev. Biochem. 55: 373-395; Uemura et al, 1988, Mol. Cell. Biol. 8:938-944). M₁ is a dsRNA that encodes a secreted toxin and immunity to the toxin (Bussey, 1988, Yeast 4:17-26) and uses the same particles as L-A for replication. Although both L-A and M₁ are affected by a variety of chromosomal genes, only three of the genes required for the maintenance of M₁ (MAK3, MAK10, and PET18) are necessary for the maintenance of L-A (see review by Wickner, 1986, supra). A deletion mutant of L-A, called X dsRNA, requires most, if not all, of the MAK genes (Esteban et al, 1988, J. Virol. 62:1278-1285).

In order to determine how both the 80 kDa and 180 kDa viral proteins can be encoded by the 4.6 kb L-A molecule and to study their differential expression, structure, function, and relation to the genetic activities of L-A, it was desirable to clone and sequence the L-A genome, such a clone not heretofore being available.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide a substantially complete cDNA clone of the L-A viral genome of S. cerevisiae.

It is another object of the present invention to provide a yeast test system for identifying factors influencing or controlling the -1 ribosomal frameshifting event that occurs during gag-pol fusion protein synthesis in retroviruses including HIV.

It is a further object of the present invention to provide a new yeast based vector for preparing particle-immobilized antigens for inducing immune response in a responsive host.

It is yet another object of the present invention to provide a stable, recombinant cDNA clone of L-A at a copy number of at least about 10,000 per cell to produce a desired protein in large amounts.

Other objects and advantages of the present invention will become evident from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 shows a summary of the overall structure of the L-A genome. The two open reading frames, ORF1 and ORF2 encode the two products of the L-A genome, the major coat protein of the viral particles and the chimeric protein having a major coat protein domain and a single-stranded RNA binding-RNA polymerase domain.

Figure 2 shows the complete sequence of the L-A genome and specific features thereof, including possible frameshifting sequences near base 2000.

Figure 3 shows the similarity between the sequences of HIV-1 and RSV that are known to produce ribosomal frameshifting and sequences in L-A in the 130 base region of overlap of ORF1 and ORF2.

Figure 4 shows the homology between L-A's ORF2 and the RNA-dependent RNA polymerase genes of a number of RNA viruses that allowed the identification of the 180 kDa protein as the L-A RNA polymerase.

Figure 5 shows the schematic design of a frameshift vector based on the frameshift sequences of the L-A virus.

Figure 6 shows constructs useful for producing particles carrying multiple copies of a protein antigen on its surface for inducing an immune response.

Figure 7 shows the use of a vector to support the replication and increase the copy number of the X dsRNA-based expression vector.

DETAILED DESCRIPTION OF THE INVENTION

The above and various other objects and advantages of the present invention are achieved by a cDNA clone of the L-A viral genome of S. cerevisiae.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference. Unless mentioned otherwise, the techniques employed herein are standard methodologies well known to one of ordinary skill in the art.

The term "substantially complete" cDNA clone, as used herein, means that the clone has the complete nucleotide sequence of the L-A viral genome except for a few bases at each end which, however, are easily repaired by using oligonucleotide mutagenesis.

The term "modified" cDNA as used herein means (1) modified to include the sequence encoding the protein whose production is desired; (2) modified to place the appropriate region with this encoding sequence downstream from a suitable promoter and upstream from a suitable transcription terminator; (3) modified in a suitable region to optimize the amount of transcript production by L-A (with the protein coding sequence); (4) modified to optimize the level of translation of the mRNA produced by L-A; (5) modified to result in secretion of the synthesized protein; (6) modified to include a multiple cloning site in an appropriate region, and the like.

MATERIALS AND METHODS

Materials

M-MLV reverse transcriptase, RNase H, and DNA polymerase I were obtained as a kit from Bethesda Research Laboratories. Exonuclease III, and S1 nuclease were from Promega Biotec. Modified T7 DNA polymerase (Sequenase) and T4 polynucleotide kinase were from United States Biochemical. Other enzymes and substrates were obtained from Bethesda Research Laboratories, New England Biolabs, New England Nuclear, LBK-Pharmacia or Boehringer Mannheim.

Saccharomyces cerevisiae strain RE59 (a arg1-1 ski2-2 L-A-HNB [D] L-BC W) was used as a source of VLPs. This strain contains a variant of L-A, L-A-HNB, which results in high levels of L-A viral particles (Uemura et al, 1988, Mol.

Cell Biol. 8:938-944). This strain has no M_1 killer dsRNA, which, if present, would strongly repress L-A replication, and the chromosomal *ski2-2* mutation also contributes to the higher yield of VLPs. *Escherichia coli* strains, DH5 α (F^- , *endA1*, *hsdR17(r^-, m^-)*, *supE44*, *thi-1*, $-$, *recA1*, *gyrA96*, *relA1*, (*argF-lacZYA*)U169, ϕ 801*lacZ* M15) and DH5 α F', were from Bethesda Research Laboratories. Plasmid SK $^+$, SK $^-$, and M13 helper phage, R408, were from Stratagene.

Construction of cDNA Clone

L-A cDNA was synthesized using (+) strand message of L-A, synthesized *in vitro*, as a template. L-A VLPs were prepared from strain RE59 as described by Fujimura et al, 1986, Proc. Natl. Acad. Sci. USA 83:4433-4437, except that the concentration of NaCl in the extraction buffer (buffer A) was raised to 500 mM to stabilize particles, and CsCl density gradient centrifugation was performed twice. L-A (+) strand RNA was synthesized from the purified VLPs as described by Welsh et al, 1980, Nucleic Acids Res. 8:2349-2363. To obtain the highest yield of the product per template in a 200 μ l reaction volume, each substrate (NTP) concentration was raised to 1.5 mM and the MgCl $_2$ concentration was adjusted to 10 mM. After 90 minutes incubation at 30°C, the reaction was stopped by phenol, and then standard phenol-chloroform extraction followed. After ethanol precipitation, the mixture of L-A ssRNA and dsRNA was used directly as a template for the cDNA synthesis without further manipulation.

The presence of dsRNA interferes neither with cDNA synthesis nor with the ligation reaction.

Two synthetic primers, GCATATGGGTAATTCCTTATCTTTTGGC (PRIMER I) and GAAAAATTTTAAATTCATATAACTCCCC (PRIMER II), were used to synthesize L-A cDNA. These primer sequences were based on the 3' and 5' terminal sequences of L-A dsRNA (Thiele et al, 1984, Mol. Cell. Biol. 4:92-100). The 5'-termini of these primers were phosphorylated by T4 polynucleotide kinase. The first strand was synthesized with m_MLV reverse transcriptase following the manufacturer's protocol, except that oligo dT was replaced by the primer I. After the first strand synthesis, the primer II was included in the reaction mixture, annealed, and the second strand synthesis was performed using DNA polymerase I with RNaseH. This use of the second primer allowed us to generate cDNA having an intact 5'-end. No labeled nucleotide was used at any point in the reaction process. The reaction was terminated by a phenol extraction and the full size cDNA which runs at the same position as dsRNA was purified from a preparative agarose gel using DEAE-cellulose paper. The size fractionated cDNA was ligated into the SmaI site of the multiple cloning site of a Bluescript vector, SK⁺. The ligated DNA mixture was transformed into frozen competent cells of E. coli strain DH5 α prepared by the method of Hanahan, 1983, J. Mol. Biol. 166:557-580. Transformants were selected on X-Gal ampicillin plates and white and pale blue

-10-

colonies were screened for their plasmid DNA inserts.

Initially 108 transformants were screened for their insert DNA size, by digesting mini-prep DNA with HindIII and BamHI. Among them, 39 clones were close to the full size. These clones were further tested by digesting the plasmids by EcoRI, EcoRV, and BamHI, and comparing the size of the fragments derived from the 5' and 3' ends of the L-A cDNA. The digested fragments were transferred from an agarose gel to two pieces of nitrocellulose paper and hybridized with ³²P end-labeled primers I and II, respectively. Among those which showed strong hybridization to both primers were L03, L05, and L28.

DNA Sequencing of L-A cDNA Clones

The plasmids, L03 and L05, have L-A cDNA inserts whose orientation is such that the 5' end of the L-A (+) strand sequence is next to the universal primer site in the SK⁺ vector, whereas the insert in L28 has the opposite orientation. These plasmids were digested at the HindIII and ApaI sites in the multiple cloning site of the SK⁺ vector, and a series of deletions were generated using ExoIII and S1 nuclease (Henikoff, 1987, Methods Enzymol. 155:156-165). ssDNAs were prepared following the protocol supplied by Stratagene with the following modifications. Fresh colonies of strain DH5αF' carrying a plasmid were inoculated into 2 ml of 2xYT medium in a 50 ml disposable plastic tube, cultured at 37°C for 2 hours, and M13 helper phage, R408, was infected

at around $\text{moi} = 10$. After vigorous shaking for two hours at 37°C , the temperature was dropped to 32°C , and the shaking was continued overnight. Although the reduction of the temperature is not essential, this method gave a consistent yield of rescued ssDNAs in a variety of deletion mutants constructed. Since F-pili are dissociated at the reduced temperature (Messing, 1983, Methods Enzymol. 101:20-78), this modification prevented re-infection by the helper phages which could eventually lyse the cells, especially those which have lost the plasmid during the overnight cultivation.

For each plasmid, about 50 deletions were sequenced using Sequenase following the manufacturer's protocol. The average reading was 400 bp. For clones in the SK⁺ vector, the M13 -20 17mer was used as a primer. Also, BamHI/HindIII fragments from 6 cDNA clones were recloned into the SK⁻ vector, and the opposite ends were sequenced using a reverse sequencing primer. The DNA sequences were assembled and analyzed using programs of UWGCG (Devereux et al, 1984, Nucleic Acids Res. 12:387-395), IDEAS, STADEN, and PIR included in the "Analysis" library at the National Cancer Institute Computer Center at Frederick, Maryland. Homology was also examined using the FASTA (Pearson et al, 1988, Proc. Natl. Acad. Sci USA 85:2444-2448) and FPAT programs provided by David Lipman, Chuck Buckler and William Pearson.

A deposit of the cDNA clone of the L-A dsRNA has been made at the ATCC, Rockville, Maryland, on February 10, 1989

under accession number 67,888. The deposit shall be viably maintained, replacing if it becomes non-viable, for the life of the patent or a period of 30 years from the date of the deposit, or for 5 years from the last date of request for a sample of the deposit, whichever is longer, and made available to the public without restriction in accordance with the provisions of the law. The Commissioner of Patents and Trademarks, upon request, shall have access to the deposit.

Among various utilities of the clone of the present invention, at least the following should be noted.

1. To identify factors influencing the ribosomal frameshifting that retroviruses, including HIV, use to make the gag-pol fusion protein which is vital to the viral replication.

2. To prepare particles that have a particular antigen protruding from multiple sites on the particle surface for use in immunization or in biological and immunological tests.

3. An RNA expression vector and as a helper for such an RNA expression vector.

These utilities are now described.

Use of cDNA clones of L-A in examining factors affecting ribosomal frameshifting.

A study of the cDNA clone of the present invention reveals that the 4579 bp L-A has two open reading frames (ORFs). ORF1 of 680 amino acids extends from base 30 to base 2072 of the L-A(+) strand and encodes the major coat protein.

A second ORF of 868 amino acids (ORF2) extends from base 1940 to 4546 and encodes part of the ssRNA-binding protein. The other part of this 180 kDa ssRNA-binding protein is encoded by ORF1. Fusion of ORF1 and ORF2, apparently by a -1 translational frameshift, produces the complete 180 kDa protein. A site similar to the site of frameshifting in HIV, RSV and other retroviruses is found within the 143 bp overlap of ORF1 and ORF2 (Fig. 3). ORF2 also contains a sequence characteristic of the RNA-dependent RNA polymerases of several picorna- and togaviruses (Fig. 4).

Retroviruses, including HIV, use ribosomal frameshifting to make a large amount of the gag protein (the major coat protein of the viral cores) and a small amount of the gag-pol fusion protein. The gag-pol fusion protein contains a number of domains, such as a domain that is identical to the gag protein itself, a domain that has the reverse transcriptase activity, and other protease, integrase and RNase H domains. The retroviruses cannot replicate unless they are able to make the host ribosomes carry out this frameshifting from the upstream gag reading frame to the downstream pol reading frame. This must be carried out while the ribosomes are in the rather small region (201 bp in the case of HIV) in which the two ORFs overlap. In all known cases, mammalian retroviruses carry out a shift of -1 base in the overlap region. There are no cases yet known in which a eukaryotic host uses ribosomal frameshifting in expressing its own

genes: Hence, this is a potential site at which the retrovirus replication cycle can be attacked. In order to carry this out, it is necessary to have a simple system in which the effect of various drugs, host factors or other perturbations on the efficiency of ribosomal frameshifting can be easily tested and determined.

As has been mentioned before, ORF1 of L-A encodes the major coat protein of the viral particles in which L-A is found in the cell (like gag of retroviruses). ORF2 encodes a part of the 180 kDa protein with homology to RNA-dependent RNA polymerases that has single-stranded RNA binding activity. Like the gag protein of retroviruses, the L-A major coat protein encoded by ORF1 is made in large amounts. Like the gag-pol fusion protein of retroviruses, the ORF1-ORF2 fusion protein (the 180 kDa protein) is made in small amounts. The mechanism appears to be same. The shift from ORF1 to ORF2 must occur in the 130 bp region of overlap between ORF1 and ORF2. The exact site of the shift in HIV, RSV and in several other retroviruses has been precisely determined and has been shown to occur at the sites shown in Fig. 3 for HIV and for RSV. The essential elements of this site are the sequences, 5' UUUUUUA 3' for HIV or 5' AAAUUUA 3' for RSV, followed closely by an inverted repeat sequence that most likely functions to slow down the ribosomes. As shown in Fig. 3, a similar sequence was found in L-A in the 130 bp overlap region. The reading frame in this homologous region

is the same as that in HIV where it frameshifts. The ORF1 frame in this G GGU CUA sequence is such that a -1 base "simultaneous slippage" (Jacks et al, 1988, Nature 331:280-283; Jacks et al, 1988, Cell, 55: 447-458) of peptidyl tRNA¹ bound to the GGU and leucyl tRNA¹ bound to the CUA leaves the non-wobble bases of each tRNA anticodon still paired to the mRNA. This mechanism has been demonstrated by Jacks et al, supra, for RSV, and is strongly suggested for other retroviruses which frameshift. L-A apparently uses the same mechanism for the synthesis of its fusion protein.

Since ribosomal frameshifting is an interaction of the ribosome and the mRNA, it is reasonable to use any message construct to examine its mechanism whether or not that construct comes from a retrovirus or not. At the time the ribosomes are frameshifting on HIV message, they have no way of knowing whether the RNA came from a retrovirus or not. The yeast system of the present invention provides easy genetic manipulability for host genes, viral components and clones.

In order to make a construct, the region at which the frameshifting occurs is placed upstream of the beta-galactosidase gene or of a gene such as LEU2 or URA3 or LYS2 or CAN1 whose increased or decreased expression can be selected (Fig. 5). The frame is adjusted so that only if the frameshifting occurs will the downstream signal gene be expressed. One or more of these constructs (Fig. 5) are

introduced into yeast strains and mutants are isolated in genes that affect frameshifting efficiency by either increasing or decreasing it. Drugs are screened for their effects on frameshifting using cells containing these "frameshift vectors". A decrease in frameshifting would interfere with retroviral replication by interfering with the supply of reverse transcriptase. Monitoring the effects on more than one construct makes the screening of effects peculiar to one signal gene a simple matter. Also, other control constructs are prepared in which the signal gene is in frame with the upstream region so that no frameshift is needed for expression. Yet other control constructs have the signal gene in the +1 frame.

Use of L-A clones to make antigen-carrying particles for Immunization

Particle-bound antigens have long been known to be more antigenic than soluble antigens (Adams et al, 1987, Nature 329:68-70). Using the process of Adams et al, gene fusions of ORF1 encoding the major coat protein of L-A are made with the gene of the protein antigen against which immunization is desired. The fusion is introduced into yeast and expressed. The particles formed by the major coat protein then have the protein antigen attached at multiple locations on each of the particles. Expression at various levels in the presence or absence of endogenous L-A virus can be used to produce particles having various numbers of antigen side chains per

particle. The DNA sequence for the protein antigen is introduced at various sites within the major coat protein gene to determine which sites give the most immunogenic particles. The particles can be purified easily using their physical properties. The particles can then be inoculated to induce the desired immune response.

Use of L-A cDNA clones to express and produce a desired protein and as a helper for an X cDNA-based RNA vector.

In the replication cycle of the L-A virus, the viral (+) strands are an intermediate. Viral (+) strands are synthesized by viral particles that contain the viral double-stranded RNA genome and extruded from the viral particles. They then serve a dual role:

(I) These (+) strands are the mRNA that is used by the ribosomes to make the two viral proteins needed for viral packaging and replication, namely, the major coat protein (80 kDa) and the major coat protein-RNA polymerase fusion protein (180kDa).

(II) The viral plus strands are encapsidated by the newly synthesized coat proteins to form new virus particles.

This natural viral replication cycle is exploited to synthesize abundant amounts of a desired protein. The L-A cDNA clone can also be modified to encode the desired protein instead of, or in addition to, the normally encoded proteins. We have defined and delimited the viral regions and signals necessary for replication, transcription and packaging of the

L-A molecule. These signals are completely included in the 5' most 25 bases of the (+) strand and the 3' most 500 bases of the (-) strand (Esteban et al, 1988, PNAS, 85:4411-4415). Replication requires the 3' terminal 30 bases and a region about 400 bases from the 3' end (the Internal Replication Enhancer). In order to produce the proteins, the region coding for the viral proteins is replaced by the gene for the desired protein. The signals for transcription and packaging and replication are left intact and the desired protein is allowed to be expressed.

Since strains having up to 20% of their total protein as major coat protein can be constructed, the transcription and translation signals normally used by the virus are quite efficient, although the method allows one to modify and improve either.

The L-A cDNA clone is altered as above to contain the desired gene and upstream of the modified L-A sequence is placed either a yeast promoter or a T7 RNA polymerase promoter. This vector is then introduced into a yeast strain that carries a normal L-A virus to supply the replication and packaging proteins. Then, the transcription of the modified L-A is turned on. The (+) strands thus produced (because they have the packaging sequences) are packaged in virus particles replicated and transcribed. The (+) strand transcripts are translated to produce the desired product.

Another use for the L-A cDNA clone in producing the

desired protein is as a source of large amounts of replication proteins. The normal L-A produces the replicase (RNA-dependent RNA polymerase) as a fusion protein with the major coat protein at about 1% of the amount of coat protein alone. While this ratio may be optimal for maintenance and replication of L-A in a normal host strain, it is probably sub-optimal for maximal copy number and expression in the ski-mutant hosts that give up to 10-20% of host proteins as L-A major coat protein. The mechanism of formation of the fusion protein is ribosomal frameshifting. This is an inefficient process and can be simply bypassed by inserting one nucleotide in the region of overlap between the two open reading frames of the L-A cDNA clone. If this clone is modified so as not to have the packaging and replication signals, the transcripts will only serve as mRNA. Thus, a large amount of RNA-dependent RNA polymerase can be supplied to the replicating virus to increase its copy number and rate of transcription.

The invention is now illustrated by the following examples:

Example 1:

This example shows the construction of a 'frameshift vector' using the frameshift region of the L-A viral sequence. The structure is schematically illustrated in Fig. 5. The yeast PYK1 promoter is linked to an AUG start codon followed by the frameshift sequence from the L-A virus. The

region of L-A analogous to HIV or RSV is shown for illustrative purposes in Fig. 5, but the exact region of L-A responsible for frame shifting is inserted during the construction. At the end of the L-A frameshift sequence is a termination codon, TAA, in the unshifted frame, so that ribosomes that have not shifted will cease translation and not have the opportunity to shift further downstream. The E. coli lacZ gene encoding beta-galactosidase, is placed next and is in the -1 frame relative to the AUG. This means that the ribosomes must shift back one base on the mRNA in order to properly translate this region. Control vectors with beta-galactosidase in the original "0" frame or in the "+1" frame are also made, but are not shown in Fig. 5. The vector also carried replication origins and selectable markers for yeast and E. coli. The vectors are transformed into a wild-type yeast strain and the levels of beta-galactosidase in the "0" frame with the "-1" frame and the "+1" frame. The amount of beta-galactosidase in a colony can also be estimated using plates containing X-gal and the blue color that results from its breakdown by the enzyme. Cells carrying the "-1" vector (the construct shown in Fig. 5) are grown on plates containing a drug to be tested for its effects on frameshifting. A change in the shade of blue can be used as a simple qualitative screen for an increase or decrease of the degree of frameshifting. Thus hundreds of drugs can be quickly screened for such an effect. More careful

quantitative assays can then be carried out on promising drugs using the simple beta-galactosidase assay of permeabilized whole cells. Since the yeast translation apparatus closely resembles that of animal cells, this method can be used to screen and determine the direct effects of drugs on the replication of retroviruses in tissue culture or in vivo tests.

The same strain carrying the frameshift vector can be mutagenized and plated for single colonies. Those colonies that are more or less blue than the parent strain can then be analyzed to detect single-gene mutations causing increased or decreased ribosomal frameshifting. The genes involved can be cloned and characterized and their mammalian counterpart examined for similar effects on translation in mammalian cells.

Example 2:

To produce particles with regions of the HIV-1 p24 protein exposed on the outer surface, constructs such as are shown in Fig. 6 are prepared. The L-A ORF1 encoding the major coat protein is fused in frame to the p24 gene. [In other constructs not shown the p24 sequence is inserted within the L-A ORF1 or at the N-terminus.] The expression vector carrying this construct is introduced into a yeast host. The major coat protein with the p24 sequences attached forms viral particles which are then purified in large amounts by conventional methods. The L-A ORF1- ORF2 fusion

protein is expressed separately from a separate vector to determine whether this fusion protein is, as we have hypothesized, necessary for particle formation (Fujimura & Wickner, 1988, Cell, 55:663-671).

The purified particles are used to induce an immune response or to generate antibody reagents. The particles are also used as the antigen in tests for determining the presence of antibody in serum of patients or animals.

Example 3:

The L-A ORF1 and ORF2 are fused in an expression vector by simply inserting one base in the region of overlap between ORF1 and ORF2. This vector then expresses the viral RNA polymerase. Along with another expression vector expressing only the major coat protein, ORFs are used to supply needed products to the X dsRNA-based expression vector which has all cis sites necessary for replication, transcription and packaging. using the cDNA clone of X, the gene for a protein whose production is desired, can be inserted, introduced into yeast, regenerated as an RNA virus and expressed by the L-A encoded transcription apparatus. The cloning of the L-A genome makes possible supplying or modifying the protein components of the L-A packaging and transcription apparatus from these clones in such a way as to optimize expression from the modified X virus-vector. This is schematically shown in Fig. 7.

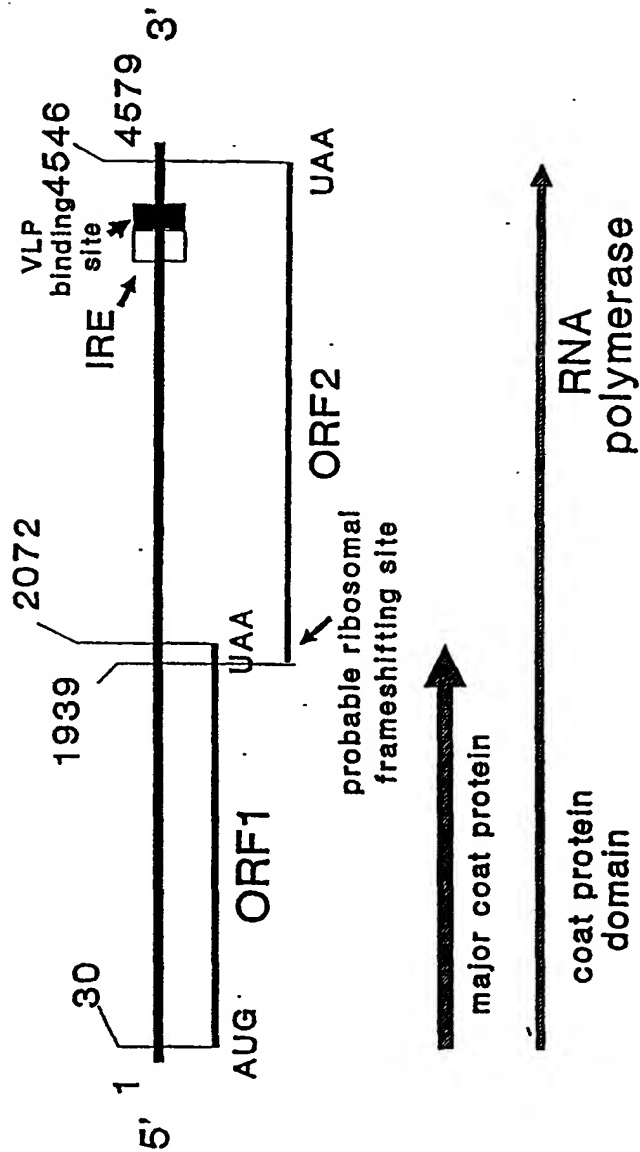
It is understood that the examples and embodiments

described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

CLAIMS

1. A DNA segment encoding a double-stranded RNA genome of the L-A virus of yeast, or a unique portion thereof.
2. The DNA segment of claim 1 having the nucleotide sequence shown in Figure 2, or a unique portion thereof.
3. The DNA segment of claim 2 wherein said unique portion of said nucleotide sequence shown in Figure 2 consists essentially of L-A virus bases numbered 1950 to 2028, inclusive.
4. The DNA segment of claim 1 consisting of a modified or unmodified cDNA clone of a double-stranded RNA genome of the L-A virus of yeast.
5. The DNA segment of claim 4 wherein said cDNA clone has the American Type Culture Collection accession number 67,888.
6. The DNA segment of claim 1 having inserted therein a sequence wherein said sequence encodes a desired protein.
8. A recombinant DNA molecule comprising said DNA segment according to claim 1 and a vector.
9. A culture of cells transformed with said recombinant DNA molecule according to claim 8.
10. A recombinant RNA molecule having a nucleotide sequence complementary to the nucleotide sequence of the recombinant DNA molecule according to claim 8.
11. A virus-like particle comprising the recombinant RNA molecule according to claim 10 and the coat protein of the L-A virus of yeast.

L-A ORFs Encode Chimeric RNA Polymerase



-21- 225 11.

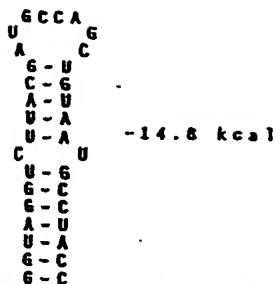
NUC 24494

NUC 24495

g2c
4001 6C6GTAACAGCSATAAAGAACAACTCGALAC6TGTCACTAAGATATTC66A6TTG6T6AT6AC6TT6T6C6C . |--- X dsRNA---> 4100
A V T A I K E Q L D K R V T K I F G V G D D V V R D I H T A H R V C
4101 GTGGCGGTATCTCGACTGATACCTGGGACCGGTTGAACTAAGATAAANCAGACAATGAAGCATATGAAATACCATACGAAATAGATGATCCATCATT 4200
G G I S T D T W A P V E T K I I T D N E A Y E I P Y E I D D P S F
4201 TTGGCCAGGGGTAAATGATTATGCTTATAAAGTCTG6AAAAATTTG6GAGAC6ACTCGAATTTAATAAGATTAAGATGCCGTAGCTAGAGGGAGTAGG 4300
M P G V N D Y A Y K V V K N F G E R L E F N K I K D A V A R G S R
4301 AGCACTATAGCTCTGAAACGTAAGGCTAGGATAACATCTAAGAGAAATGATTCGCTAACAAGTCGGAATGGGAAAGGACAATGTACAAAGCCTATAAGG 4400
S T I A L K R K A R I T S K K N E F A N K S E V E R T H Y K A Y K G
4401 GTTTGGCAGTCTCATACTATGCTAACCTGAGCAATTCATGAGTATACCACCAATGGCGAACATTGAATTTGGGCAAGCTAGATATGCTATGCAAGCAGC 4500
L A V S Y Y A N L S K F M S I P P N A N I E F G Q A R Y A M Q A A
4501 CCTTGATAGTTCTGATCCACTCCGGGCAATTACAGGTCATACTGTAAATGCCAAAAGATAATG66AATTACCCATATGC 4579
L D S S D P L R A L Q V I L * <-----Primer I----->

3

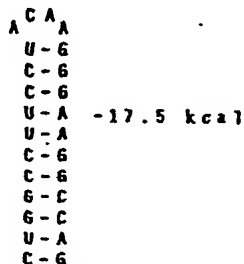
L-A



-14.8 kcal

66GUAGU6U6CGUCGUACUCAGCA66GUUAGGAGU-66AGAACCUACAG
 Major Coat Prot = ORF1-->G1RG1yLeuG1yVa1
 G1yPheArgSer-->ORF2=RNA polymerase

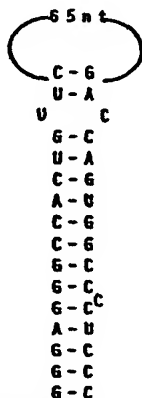
HIV-1



-17.5 kcal

666GCTAAUUUUUUUAGG6AAGAU-6GAAU
 gag ORF-->AsnPheLeuG1yArg
 TerPhePheArgGlu-->pol ORF

RSV



CCGCUUGACAAAUUUUAU-UGAAGGUAAA
 gag-->ArgLeuThrAsnLeuTer
 IleG1y...-->pol

FMD 2024 FLKDEIRPMEKIVRAIGKTRIIDVLPMEHILY.TKMMIGRFCQMHSSNNGPQIGSAVGCNPDVDWQ
 EMC 1987 FLKDELRLPIEKVQAATRIIVDVPIPIFEHCIL.GRQLLGKFASKFQTPIGLELGSALGCDPPDVAWT
 Rhino14 1876 YIKDELRSVDKVRLLGKSRLLIEASSLINDSVN.MRMKLGNLYKAFHQNPGLTGSAVGCDPPDVFS
 Hepat A 1034 CPKDELRLPIEKVLESKTRAIIDACPILDYSIL.CRMYMGPASISYFHLNPGFHTGVAIGIDPPDRQWD
 Polio 1903 YVKDELRSKTKVEQGKSRLLIEASSLINDSVN.MRMALGNLYAAAFHKNPGLVITGSAVGCDPPDLFWS
 CoxsacB3 157 HYKDELRSIEKVAKGSRLIEASSLINDSVN.MRQTIFGNLYKTFFHLNPGVVTGSAVGCDPPDLFWS
 L-A ORF2 413 PPEVRAWLSTKYEWMQKQRAIYGTDLRSTLITNFAMERCEDVLTHKFPVGDQAEAAKVKHRYNNM

Region I

Carnat 524 RYITKEGCRMSGDMNTALGNCLLACLITKH.....LMKIRSRRLINNGDDCVLI
 TobEch 2580 IIKKHKGNNSGQPSITVVDNTLMVLIAMLY.....TCEKCGINKEEIVYVNGDDLLIA
 Mdlbrg 804 RFKFGAMMKSGMFLTILFVNTMLNMTIAS.....RYLEERLTNSKCAAFIGDDNIVH
 WNIleF 3120 VIISREDIQRSGQVVTYALNTFTNLAVQKV.21 aa.GPKVRTWLFENGEEERLSRMAYSGDDCVVK
 YellowF 3100 VIISREDIQRSGQVVTYALNTITNLKVQLI.21 aa.LTRLEAWLTEHGCDRLKRMAYSGDDCVVR
 Rhino14 2006 IYVVEGMPSPGCSGTSIFNSMINNIIRT.....LILDAYKGIDLD.KLKILAYGDDLIYS
 Hepat A 1191 CYHYCGSMPSGSPCTALLNSIINNVLNY.....VFSKIFGKSPVFFCQALKILCYGDDVLIIF
 Polio 2025 TYICVKGGMPSGCSGTSIFNSMINNIIRT.....LLKTYKGIDLD.HLKMIAYGDDVIAS
 Coxsac B 300 HYFVRGGMPSGCSGTSIFNSMINNIIRT.....LMLKVYKGIDLD.QFRMIAYGDDVIAS
 L-A ORF2 541 WYRLQGTLLSGWRLLITFMNTJVLNWA.....YMKLAGVFDLDDVDQSDSVHNQDDVMS

Region II

Region III

ig 5

Frameshift Vector Based on the L-A Virus

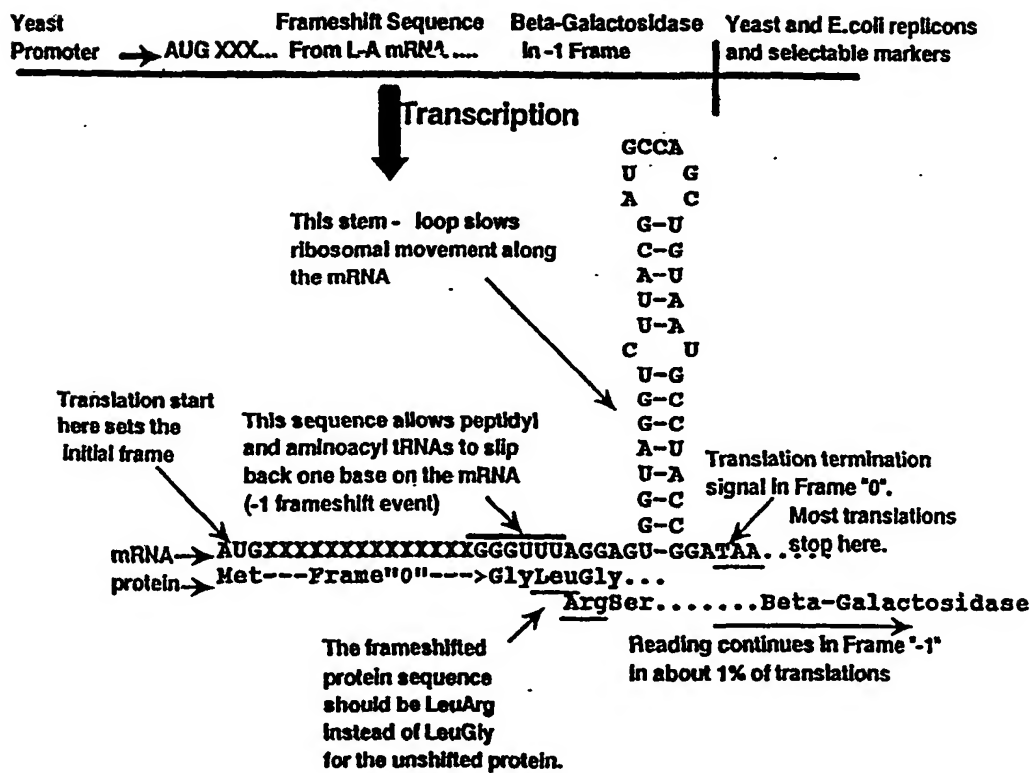
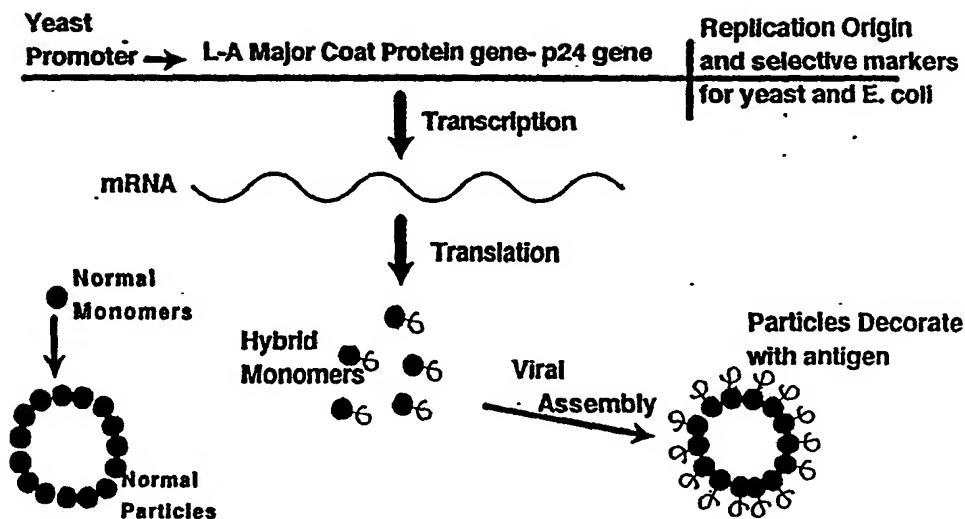
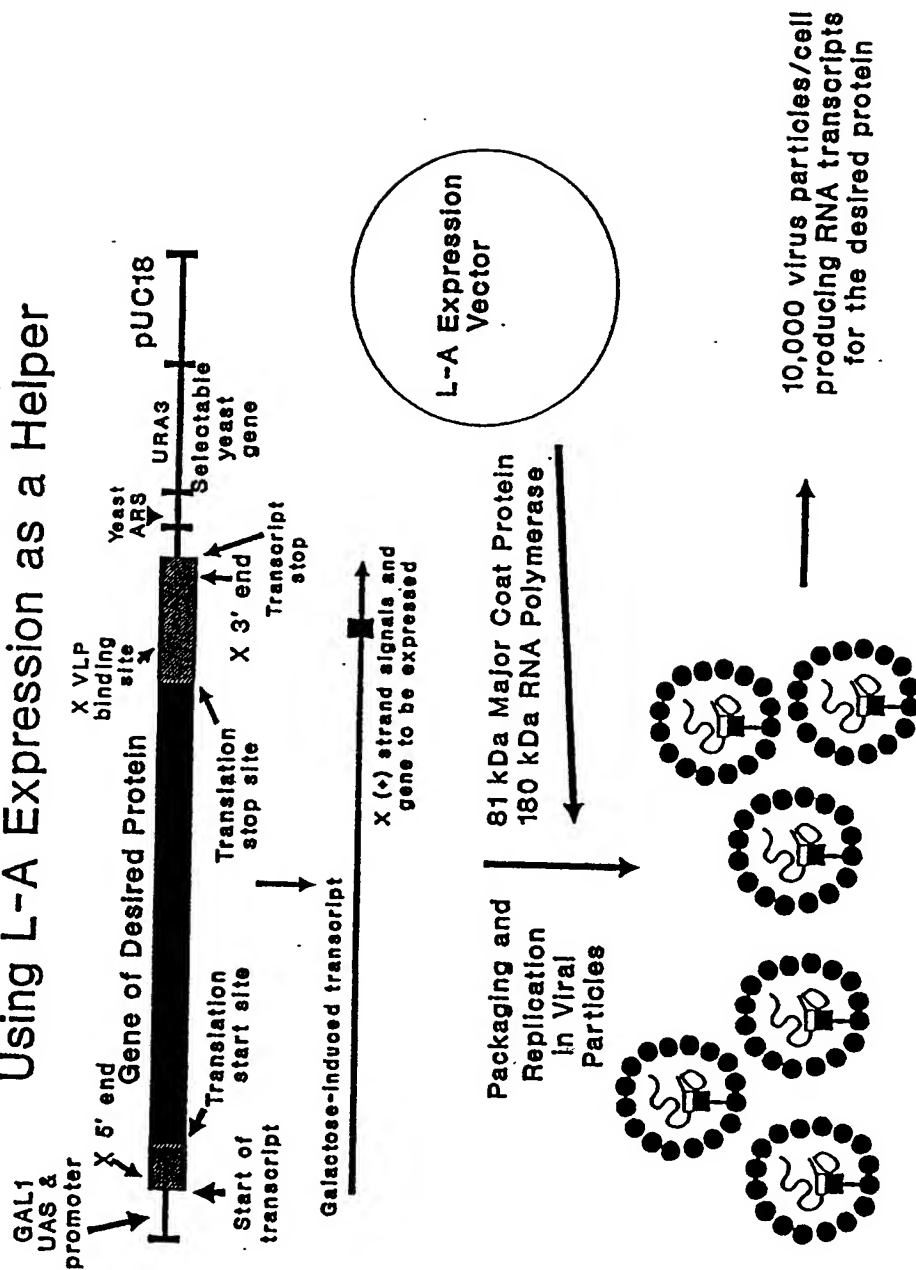


fig. 6



RNA VIRUS VECTOR Using L-A Expression as a Helper



INTERNATIONAL SEARCH REPORT

International Application No

PCT/US90/01716

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): - C12N 15/00, 7/00, 5/00; C07H 15/12		
US CL.: 435/172.3, 235, 255, 320; 536/27		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
U.S.	435/235, 255, 320, 172.3; 536/27	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched *		
Dialog [(CAS File 1967-1990)(Biotech File 1967-1990)]		
III. DOCUMENTS CONSIDERED TO BE RELEVANT 14		
Category *	Citation of Document, 15 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
Y	Yeast, Volume 4, special issue, published August 1988. W. Wilson, et al. "Human Immunodeficiency virus (HIV-1) gene expression: ribosomal frameshifting requires a short sequence and no secondary structure in yeast and mammalian systems" pp. S177. see entire abstract.	1-11
X Y	Mol. Cell. Biol., Volume 4, Number 1, published January 1984. D.J. Thiele, et al. "Multiple L double-stranded RNA species of <i>Saccharomyces cerevisiae</i> : evidence for separate encapsidation" pp. 92-100. see entire article.	1-3,6,8 4,5,9-11
Y	Cell, Volume 55, published 18 November 1988. T. Fujimura, et al. "Gene overlap results in a viral protein having an RNA binding domain and a major coat protein domain" pp. 663-671. see entire article.	1-11
<p>* Special categories of cited documents: 16</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"Z" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search *	Date of Mailing of this International Search Report *	
27 June 1990	01 AUG 1990	
International Searching Authority *	Signature of Authorized Officer	
ISA/US	<p>for Beth A. Burrows</p> <p>NGOC-HO</p> <p>INTERNATIONAL DIVISION</p>	

Form PCT/ISA/210 (second sheet) (May 1986)

NUC 24502

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, ¹² with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y	Biological Abstracts, Volume 86, issued 15 September 1988. R. Esteban, et al. "Site-specific binding of viral plus single-stranded RNA to replicase-containing open virus-like particles of yeast" see Ref. No. 58368, Proc. Natl. Acad. Sci. USA 85(12):4411-4415.	1-11
<u>X</u> Y	GenBank and UEMBL nucleotide sequence data bases. Sequence provided to GenBank data base on 08 September 1988 by R.B. Wickner. (93% sequence homology with nucleotides 4041 to 4500 of the instant application)	<u>1,2,4,8</u> 3,5,6,9-11
Y	Nucl. Acid. Res., volume 13, Number 5, published April 1985. J. Bruenn, et al. "Long internal inverted repeat in a yeast viral double-stranded RNA" pp. 1575-1591. see entire article.	1-11

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Cell, Volume 55, published 23 December 1988. W. Wilson, et al. "Ribosomal frameshifting is directed by a short sequence in both mammalian and yeast systems" pp. 1159-1169. see entire article.	1-11
X,P	J. Biol. Chem., Volume 264, published 25 April 1989 T. Icho, et al. "The double-stranded RNA genome of yeast virus encodes its own putative RNA polymerase by fusing two open reading frames" pp. 6716-6723. see entire article.	1-11

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out², specifically:
3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING³

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.



Paul N. Kokulis

11/08/2004 05:20 PM

To: "Richard Meserve" <rmeserve@pst.ciw.edu>
cc: john <jlively@pst.ciw.edu>
Subject: Re: Diamond Innovations

Dear Dick

Thanks for the note. I called Singer as a follow-up to your note but he was away for the day and I could only leave a message. He is expected back tomorrow and hopefully he will return my call as I left a reasonably detailed message..

I will keep you posted.

Paul

Paul N. Kokulis
Morgan, Lewis & Bockius LLP
1111 Pennsylvania Avenue NW
Washington, DC 20004
PKokulis@morganlewis.com
202.739.5455 (voice)
202.739.3001 (fax)

"Richard Meserve" <rmeserve@pst.ciw.edu>



"Richard Meserve"
<rmeserve@pst.ciw.edu>
u>

11/06/2004 08:02 AM

To: "Paul Kokulis" <pkokulis@morganlewis.com>
cc: john <jlively@pst.ciw.edu>
Subject: Diamond Innovations

Paul --

This note is to give an OK for your efforts to determine whether a meeting with Diamond Innovations would be fruitful along the lines of your message. I will be away from Washington until Wednesday night and may not be in e-mail contact until then.

Dick

Richard A. Meserve
President
Carnegie Institution of Washington
1530 P St., NW
Washington, DC 20005
(202) 387-6404

NUC 24505